neuroReport

Website publication 13 July 1997

NeuroReport 9, 2523–2525 (1998)

Spinal κ_1 and κ_2 opioid binding sites in rats, guinea pigs, monkeys and humans

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SEVERAL lines of work demonstrate that there are two subtypes of κ opioid receptors. Intrathecally administered agonists for the κ_1 subtype are not effective in treating pain, whereas agonists for the κ_2 receptor are anti-hyperalgesic and anti-allodynic. The question addressed here was whether the ratio of spinal κ_1 to κ_2 receptors was conserved across species. Thus, binding experiments were performed on spinal cord membranes from rats, guinea pigs, monkeys and humans. We found that κ_2 receptors were approximately ten times more abundant than κ_1 receptors in all species tested. This suggests that the anti-hyperalgesic and anti-allodynic properties of κ_2 agonists may also be conserved. Therefore, selective κ_2 agonists may be effective in treating chronic pain in humans. *NeuroReport* 9: 2523–2525 © 1998 Rapid Science Ltd.

Key words: Allodynia; Analgesia; Hyperalgesia; Kappa opioid receptors; Spinal cord

Introduction

Recent evidence indicates that there are at least two receptors that can be operationally classified as κ opioid receptors. These receptors are functionally and pharmacologically distinct,[1,2] but only one has been cloned. The cloned receptor was labelled the κ_1 receptor and compounds such as *N*-methyl-*N*[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-benzeneacetamide (U69,593) and 3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexl]-benzeneacetamide methanesulfonate (U50,488) are highly selective ligands for this receptor.[3] The second κ opioid receptor, which is not molecularly characterized, was labelled the κ_2 receptor. U69,593 and U50,488 bind to the κ_2 receptor with very low affinity.[1] However, less selective κ agonists such as bremazocine and methyl 4-[3,4-dichlorophenylacetyl]-3-[(1-pyrrolidinyl)methyl]-1-piperazinecarboxylate (GR89,696) bind with relatively high affinity.[1] Hence, these differences in binding are used to operationally define the two receptor subtypes. Physiologically, κ_1 receptors inhibit glutamate release from pre-synaptic terminals while κ_2 receptors post-synaptically inhibit the function of NMDA receptors.[2,4–8]

Behavioral characterization of the two subclasses of κ receptors in rat models of persistent pain demonstrated that κ_1 receptor agonists were ineffective as analgesics when given spinally. Agonists that act at κ_2 receptors, however, were very effective at blocking the enhanced sensation of pain (hyperalgesia) and lowered pain threshold (allodynia) associated with an injury.[9] The anti-hyperalgesic and anti-allodynic properties of κ_2 agonists contrast sharply with traditional opioid analgesics, which inhibit all sensory function. These findings suggest that κ_2 agonists may be effective at inhibiting only the symptoms associated with the pathological state while leaving normal function intact. Such symptom-specific actions would be particularly useful for the treatment of chronic pain in humans.

To further evaluate the potential of κ_2 agonists as therapeutics for chronic pain in humans, we conducted binding experiments on membranes from the spinal cords of several species, including humans, to determine the relative abundance of κ_1 and κ_2 receptors. These experiments demonstrate that the ratio of κ_1 to κ_2 receptors is conserved in the spinal cords of several species.

Materials and Methods

Post-mortem human spinal cord tissue was obtained through the anatomical gift act from The Delaware

Valley Transplant Authority (KidneyOne). All human tissue was harvested within 6 h of death.

Animal spinal cord tissue was collected under protocols approved by the Animal Care and Use Committee of the National Institute of Dental Research. These experiments comply with the National Institutes of Health guide for care and use of laboratory animals. Every effort was made to use the minimum number of animals possible.

Spinal cord membranes were prepared as previously described.[1,6] Briefly, spinal cords were removed from euthanized (100 mg/kg pentobarbital, i.p. or i.v.) rats, guinea pigs, monkeys (*Macaca mulatta* and *M. fascicularis*) or postmortem humans, frozen on dry ice and then stored at –70°C for at least 1 week. The tissue was then homogenized in 10 ml/g of 50 mM Tris (pH 7.4) and centrifuged at $10\,000 \times g$ (4°C) for 30 min. The membranes were then resuspended in Tris buffer and stored at –70°C until needed. Each assay tube contained 0.5 mg membrane protein, varying concentrations of either [^{3}H]bremazocine or [^{3}H]U69,593 and the appropriate blocking ligands in 50 mM Tris buffer (pH 7.4). The final volume of each assay tube was 1 ml. All assays were performed in triplicate and each experiment was repeated 2–4 times. In the κ_2 binding experiments [^{3}H]bremazocine was used as the radioligand and [D-Ala,N-Me-Phe,Gly-ol]-enkephalin (DAMGO) (1 μM), [D-Pen^{2,5}]-enkephalin (DPDPE) (1 μM) and U69,593 (1 μM) were used to block μ, δ and κ_1 opioid receptors respectively. For the κ_1 experiments [^{3}H]U69,593 was used as the radioligand and no blocking agents were used. Non-specific binding was defined using 10 μM GR89,696 or 10 μM naloxone. Assay tubes were incubated for 90min at room temperature, filtered through Whatman GF/C filters and washed three times with 5 ml ice-cold Tris buffer. The filters were presoaked in 0.5% polyethlenimine and then counted in a scintillation counter. The affinity of the radioligands (K_d) and the number of bindings sites (B_{max}) were calculated from the saturation binding curves using the statistical software PRISM (Graphpad Software inc., San Diego, CA).[1] [^{3}H]U69,593 and [^{3}H]bremazocine were purchased from Dupont NEN (Boston, MA). DAMGO, DPDPE, GR89,696 and U69,593 were purchased from Research Biochemicals Inc. (Natick, MA). Naloxone was purchased from Sigma (St. Louis, MO).

Results

The binding of [^{3}H]U69,593 to κ_1 opioid receptors in the spinal cord of rats, guinea pigs, monkeys and humans was very similar. The B_{max} for all species examined was between 15 and 55 fmol/mg protein (Table 1). Except for the K_d of U69,593 in the monkey, the values of K_d were all in the low nanomolar range (Table 2). The affinity of U69,593 in monkey spinal cord was 11.5 ± 2.9 nM.

The binding of [3H]-bremazocine to κ_2 opioid receptors in the presence of μ, δ and κ_1 opioid receptor blocking agents was also similar in rats, guinea pigs, monkeys and humans. However, values of B_{max} were ~10-fold higher for bremazocine (ANOVA; $F_{1,6} = 21.01$, $p = 0.004$) than for U69,593 (Table 1) and values for K_d were between 10 and 30 nM (Table 2). As illustrated by the saturation binding curves for human tissue (Fig. 1), the curves were fit well by single binding site models. In the case of bremazocine, saturation of the κ_2 binding sites was not achieved because the use of higher concentrations of

Table 1. Cross-species comparison of binding affinities (K_d; mean ± s.e.m.) of U69,593 and bremazocine to spinal cord membranes.

Species	U69,593	Bremazocine
Rat	4.10 ± 1.30	8.30 ± 5.40
Guinea pig	4.20 ± 0.90	17.0 ± 3.50
Monkey	11.5 ± 2.90	27.9 ± 14.1
Human	5.40 ± 1.00	18.7 ± 6.10

Table 2. Cross species comparison of the mean number (± s.e.m.) of kappa$_1$ and kappa$_2$ binding sites in spinal cord membranes (B_{max}, fmol/mg protein).

Species	U69,593	Bremazocine
Rat	15.9 ± 1.90	110.0 ± 56.0
Guinea pig	31.8 ± 3.30	189.1 ± 56.5
Monkey	17.4 ± 4.30	230.6 ± 183
Human	54.3 ± 22.7	284.8 ± 53.6

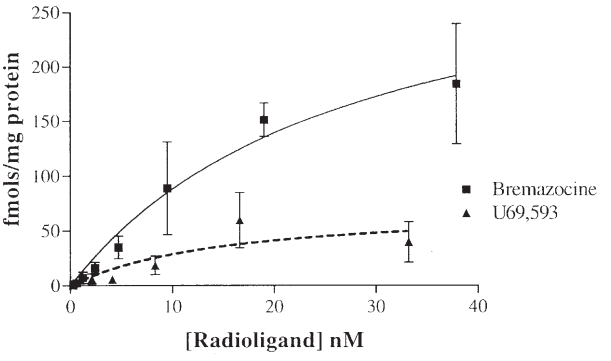

FIG. 1. Comparison of kappa$_1$ and kappa$_2$ receptor binding in human spinal cord membranes. Saturation binding experiments were carried out as described in Materials and Methods using [^{3}H]U69,593 and [^{3}H]bremazocine as the radioligands for κ_1 and κ_2 receptors respectively. The binding data was then fit with one site binding models using the program PRISM (Graphpad Software inc., San Diego, CA). The correlations were $r^2 = 0.7703$ and $r = 20.9813$ for κ_1 and κ_2 binding respectively. κ_2 binding sites were ~ 10 times more abundant than κ_1 binding site in human spinal cord membranes. These findings are similar to those found with all other species tested.

the radioligand would displace the blocking agents from non-κ_2 binding sites. Higher concentrations of U69,593 were not needed because saturation was achieved with concentrations of approximately 33 nM. For all species the fitted curves were highly correlated with the saturation binding data ($r^2 > 0.7$).

Discussion

The goal of this study was to quantify κ opioid receptor subtypes in the spinal cord of several species, including humans. In rats, guinea pigs, monkeys and humans we demonstrated that κ_2 opioid receptors are ~10-fold more abundant than κ_1 opioid receptors. In a previous study, the number of κ_1 and κ_2 receptors in the brain varied significantly from species to species.[10] Thus, the similarities in the spinal cord are somewhat surprising, but encouraging. Activation of spinal κ_2 opioid receptors in rats results in the inhibition of hyperalgesia and allodynia. The similarity in spinal κ opioid receptor binding across species suggests that κ_2 agonists may be anti-hyperalgesic and anti-allodynic in other species as well. These findings provide an incentive for the development of highly selective κ_2 agonists for trials in humans sufferings from chronic pain.

The mechanism for κ_2 receptor regulation of hyperalgesia and allodynia is not fully understood. It is interesting to note, however, that NMDA receptor antagonists are also anti-hyperalgesic and anti-allodynic when injected intrathecally[11] and that κ_2 opioid receptors were found to inhibit NMDA receptor function *in vitro*.[1,2] Thus, the hypothesis we proposed was that the anti-hyperalgesic and anti-allodynic properties of κ_2 receptors are mediated by the receptor's ability to regulate NMDA receptor function.[9] However, this hypothesis remains to be tested.

Currently, there is some controversy over the existence of κ opioid receptor subtypes. Several binding, physiological and behavioral studies have demonstrated multiple κ receptor subtypes.[1,2,9,10,12] However, only one κ opioid receptor has been cloned to date and this clone is pharmacologically identical to the κ_1 receptor.[3] In spite of the numerous attempts to clone other κ opioid receptor subtypes, no molecular evidence has been presented to demonstrate their existence. Therefore, the κ_2 receptor remains a pharmacologically defined site. Because of the current lack of molecular support for multiple κ opioid receptors, it does not appear that the κ_2 receptor is a splice variant of the cloned receptor. It is possible, however, that the κ_2 receptor represents a post-translational modification of the κ_1 receptor. In such a scenario, cloning techniques would not recognize the novel receptor form. Alternatively, the κ_2 receptor may represent a novel type of opioid receptor that is evolutionarily convergent with the cloned opioid receptors. In any event, the lack of molecular support for κ_2 receptors does not detract from the potential pharmacological significance of the site for the treatment of chronic pain.

Conclusion

Chronic or persistent pain affects large numbers of people worldwide, and hyperalgesia and allodynia represent two symptoms that most significantly influence the quality of life for these people. However, effective treatments for these symptoms are not currently available. The results of the present study suggest that κ_2 agonists may perform as well as humans as they do in rats. Thus, κ_2 receptor agonists represent a relatively new, and promising, line of attack in the treatment of chronic or persistent pain.

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Received 6 May 1998; accepted 19 May 1998